

# Chromosomal mapping and quantitative analysis of estrogen-related receptor alpha-1, estrogen receptors alpha and beta and progesterone receptor in the bovine mammary gland

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## Abstract

Steroid receptors are key transcriptional regulators of mammary growth, development and lactation. Expression of estrogen receptors alpha (ER $\alpha$ ) and beta (ER $\beta$ ), progesterone receptor (PR), and estrogen-related receptor alpha-1 (ERR $\alpha$ ) have been evaluated in bovine mammary gland. The ERR $\alpha$  is an orphan receptor that, in other species and tissues, appears to function in the regulation of estrogen-response genes including lactoferrin and medium chain acyl-CoA dehydrogenase and in mitochondrial biogenesis. Expression of ER $\alpha$ , ER $\beta$ , PR and ERR $\alpha$  was characterized in mammary tissue obtained from multiple stages of bovine mammary gland development using quantitative real-time RT-PCR. Expression was evaluated in prepubertal heifers, primigravid cows, lactating non-pregnant cows, lactating pregnant cows and non-lactating pregnant cows ( $n=4$  to 9 animals/stage). In

addition, ER $\alpha$ , ER $\beta$ , PR and ERR $\alpha$  were mapped to chromosomes 9, 10, 15 and 29 respectively, by linkage and radiation hybrid mapping. Results indicated that expression of ER $\alpha$ , PR and ERR $\alpha$  was largely coordinately regulated and they were present in significant quantity during all physiological stages evaluated. In contrast, ER $\beta$  transcripts were present at a very low concentration during all stages. Furthermore, no ER $\beta$  protein could be detected in bovine mammary tissue by immunohistochemistry. The ER $\alpha$  and PR proteins were detected during all physiological states, including lactation. Our results demonstrate the presence of ER $\alpha$ , PR and ERR $\alpha$  during all physiological stages, and suggest a functional role for ERR $\alpha$  and a relative lack of a role for ER $\beta$  in bovine mammary gland development and lactation.

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## Introduction

Ovarian steroids are important regulators of mammary growth, development and lactation. Their influence on mammary development in numerous species has long been appreciated. Ovariectomy of calves prevents mammary growth, which can be restored by estrogen administration (Wallace 1953). Studies in rodents have confirmed these observations and demonstrated that estrogens are required for growth and morphogenesis of mammary ducts. Classical endocrine ablation and hormone replacement studies in rats (Lyons 1958) and recent estrogen receptor (ER) knockout studies in mice (Bocchinfuso & Korach 1997) indicated that estrogens are essential for the normal mammary ductal growth that occurs from birth to sexual maturity. In sexually mature animals, progesterone is necessary for the lobulo-alveolar development that

occurs during pregnancy (Lyons 1958, Briskin *et al.* 1998). During lactogenesis, a reduction in circulating levels of progesterone is necessary for induction of copious milk secretion, because progesterone inhibits effective induction of  $\alpha$ -lactalbumin synthesis and initiation of stage II lactogenesis by lactogenic hormones (Turkington & Hill 1969, Capuco & Tucker 1980, Shamay *et al.* 1987). Although estrogen may enhance lactogenesis, it is generally thought to inhibit lactation by reducing milk secretion (Bruce & Ramirez 1970) and possibly by increasing mammary regression (Capuco *et al.* 2003).

The varied effects of estrogens and progesterone on the mammary gland are typically mediated by specific steroid receptors (Athie *et al.* 1996). However, the action of steroid receptors involves extensive interaction among nuclear receptors, their various isoforms, co-regulator proteins, and other hormones and growth

factors (Saunders 1998, Feldman *et al.* 1999, Yee & Lee 2000, Conneely 2001, McDonnell & Norris 2002, Tremblay & Giguere 2002, Conneely *et al.* 2003). The estrogen receptor exists as two isoforms encoded by separate genes, the classical ER $\alpha$  (Jensen & Jacobsen 1962, Greene *et al.* 1986) and the more recently cloned and characterized ER $\beta$  (Kuiper *et al.* 1996). Expression of both ER isoforms has been reported in mammary tissue of a variety of species. Progesterone receptor (PR) is encoded by a single gene, but exists as two isoforms, A and B, that are the result of transcription initiation from two promoters (Kastner *et al.* 1990). We have previously described the presence of ER and PR in mammary tissue of Holstein calves and their co-localization in a subpopulation of epithelial cells (Capuco *et al.* 2002a) and in cows during the periparturient period (Capuco *et al.* 1982). Although we demonstrated the presence of PR in lactating bovine mammary gland by ligand binding methodology (Capuco *et al.* 1982), it is commonly stated that lactating tissue does not contain PR. A recent study has evaluated changes in ER and PR expression in the bovine mammary gland (Schams *et al.* 2003). Much remains to be learned about the regulation of ER and PR in the bovine gland, despite their importance to mammary gland function.

Estrogen-related receptor alpha-1 (ERR $\alpha$ ) is a member of the group III nuclear receptor superfamily with extensive sequence homology with the estrogen receptors (Giguere *et al.* 1988, Giguere 1999). The ERR $\alpha$  is classified as an orphan receptor, with no known endogenous ligand. However, the quantity and activity of ERR $\alpha$  can be regulated by estrogen and ERR $\alpha$  may play a role in the regulation of estrogen responsive genes (Shigeta *et al.* 1997b, Bai & Giguere 2003). Of particular impact on mammary gland biology, ERR $\alpha$  appears to play a role in the regulation of lactoferrin, medium-chain acyl-CoA dehydrogenase and thyroid hormone receptor alpha gene activation (Zhang & Teng 2000, Giguere 2002). Expression of ERR $\alpha$  has been reported for a number of tissues in humans and rodents, and we have detected a significant quantity of transcripts in bovine mammary tissue (A V Capuco, unpublished data).

The objectives of this study were to characterize expression of ER $\alpha$ , ER $\beta$ , PR and ERR $\alpha$  throughout development and in different functional states of the bovine mammary gland and to map these genes on the bovine genome. Preliminary data were previously reported by Connor *et al.* (2003a,b).

## Materials and Methods

### *Animals and tissue sampling*

Mammary tissues used in this study were obtained from Holstein cattle at various stages of development: (1) 3-month-old heifers,  $n=4$ , (2) primigravid cows,  $n=9$ , (3) lactating multiparous non-pregnant cows,  $n=5$ , (4)

lactating multiparous pregnant cows,  $n=5$  and (5) non-lactating cows, 25 days after cessation of milking and 35 days before expected parturition,  $n=5$ . Mammary tissue from primigravid cows was obtained by biopsy (Farr *et al.* 1996, Capuco *et al.* 2001) and remaining tissues were obtained at the time of slaughter at the Beltsville Agricultural Research Center (BARC) abattoir. Use of animals for these investigations was approved by the BARC Animal Care and Use Committee.

Portions of mammary tissues obtained by biopsy or at slaughter were frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until RNA extraction. Other portions of tissues were fixed overnight in 10% neutral buffered formalin at  $4^{\circ}\text{C}$  and then stored in 70% ethanol until dehydration and embedding for immunohistochemistry (IHC).

### *Immunohistochemistry*

After fixation, tissues were processed for IHC as described previously (Capuco *et al.* 2002a), except that tissue sections labeled with primary antibody were stained using the Picture-Plus kit (Zymed Laboratories, San Francisco, CA, USA) according to the manufacturer's recommended protocol.

Primary antibody for localization of ER $\alpha$  was mouse monoclonal antibody C-311 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), used at a concentration of  $2\text{ }\mu\text{g/ml}$ . The primary antibody used for localization of ER $\beta$  was rabbit polyclonal antibody PA1-311 (Affinity BioReagents, Golden, CO, USA), used at a concentration of  $4\text{ }\mu\text{g/ml}$ . The primary antibody for PR localization was mouse monoclonal antibody clone PR-AT4-14 (Research Diagnostics Products Inc., Flanders, NJ, USA), which recognizes both A and B forms of PR and was used at a concentration of  $5\text{ }\mu\text{g/ml}$ .

Tissue sections were photographed with a Spot digital camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA) on a Zeiss Axioskop microscope (Carl Zeiss Inc., Thornwood, NY, USA). To obtain grayscale images with suitable contrast of diaminobenzidine staining, image files were filtered using the orthochromatic filter in Iridius 1.1 Actions for Photoshop (Iridius, Daventry, Northamptonshire, UK) using Photoshop 7.0 (Adobe Systems Incorporated, San Jose, CA, USA).

### *RNA preparation and real-time RT-PCR*

Total RNA was isolated using RNeasy isolation kits with on-column DNase digestion (Qiagen Inc., Valencia, CA, USA). The RNA quality was evaluated and concentration determined using the Agilent 2100 Bioanalyzer with RNA 6000 Nano LabChip kits (Agilent Technologies, Palo Alto, CA, USA).

Reverse transcription was performed using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). Purified RNA was diluted to a concentration of  $35\text{ ng}/\mu\text{l}$ . Five-hundred and twenty-five nanograms RNA

**Table 1** Summary of gene targets evaluated by quantitative real-time RT-PCR

Gene	Fragment size (bp)	Sense primer (5' → 3')	Antisense priemr (5' → 3')	Annealing temperature (°C)	PCR efficiency (%)	Correlation coefficient (r)	Mean CV (%)
ER $\alpha$	148	TTGCTGGCTACTTCGTCTC	GGTGGATGTGGTCCCTCTC	54.0	95.3	1.000	0.87
ER $\beta$	128	ACCTGCTGAATGCTGTGAC	GTTACTGGCGTGCCTGAC	54.0	95.2	0.999	0.41
PR	116	CAGTGGTCAAGTGGTCTAAATC	TCTCCATCCTAGTCCAAATACC	54.0	91.7	1.000	1.07
ERR $\alpha$	144	GCCTCCAACGAGTGTGAG	CCGCCGCTTGTACTTCTG	54.0	91.9	0.998	0.84

CV, coefficient of variation.

were used per 20- $\mu$ l reaction volume. For each first strand synthesis reaction, a parallel control reaction was performed in the absence of reverse transcriptase enzyme. Incubation conditions were those suggested by the manufacturer: 25 °C for 5 min, 42 °C for 30 min, 85 °C for 5 min.

Following reverse transcription, 2  $\mu$ l cDNA reaction product were used per 25  $\mu$ l of real-time PCR reaction employing SYBR green detection. Primers were designed using Beacon Designer 2.1 software (Bio-Rad Laboratories) based on bovine mRNA sequences available in GenBank (U64962 (ER $\alpha$ ), AF110402 (ER $\beta$ ), AJ557823 (PR)) or tentative consensus sequences in the *Bos taurus* Gene Index (<http://www.tigr.org/>; TC180604 (ERR $\alpha$ )). Primer sequences and annealing temperature used for each gene target are presented in Table 1. Real-time PCR was performed in the Bio-Rad iCycler using iQ SYBR Green Supermix (Bio-Rad Laboratories). PCR amplicons were cloned into pCR4-TOPO cloning vector (Invitrogen Corp., Carlsbad, CA, USA) and sequenced using a CEQ8000 automated sequencer and DTCS Quickstart Chemistry (Beckman Coulter, Fullerton, CA, USA). The nucleotide sequence of each gene target was confirmed against previously reported bovine sequences.

Standards were prepared from PCR amplicons purified using the QIAquick purification kit (Qiagen Inc.). Product concentrations were determined using the Agilent 2100 BioAnalyzer and DNA 500 kits (Agilent Technologies) and diluted to contain  $1 \times 10^2$  to  $1 \times 10^8$  molecules per  $\mu$ l. The quantity of cDNA in unknown samples was calculated from the appropriate external standard curve run simultaneously with samples. Samples and standards were assayed in duplicate. Assay performance for each gene is reported in Table 1.

#### Linkage and radiation hybrid mapping

Primers designed for PCR amplification of genomic DNA were based on bovine sequences of ER $\alpha$ , ER $\beta$ , PR and ERR $\alpha$  available in GenBank and the *Bos taurus* Gene Index (accession numbers U64962, NM\_174051, AJ557823, and TC196217 respectively). Primers were

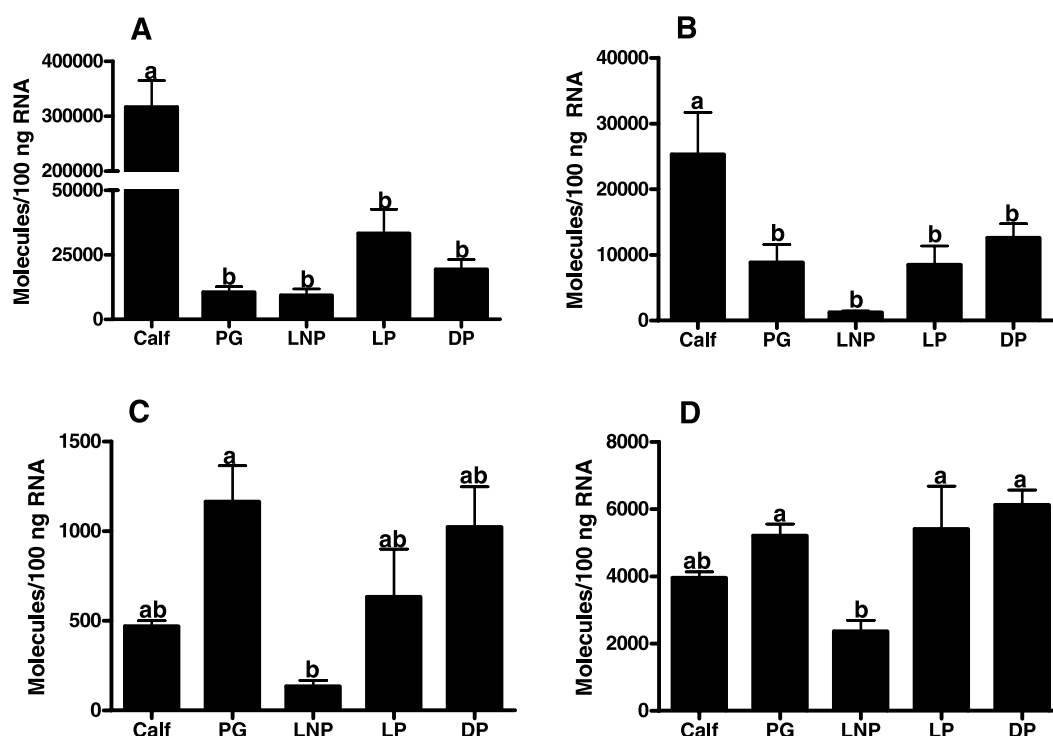
positioned within consecutive exons to PCR amplify the intervening intron and produce an amplicon <5 kb, based on human gene structure information available in the NCBI LocusLink Evidence Viewer (<http://www.ncbi.nlm.nih.gov/LocusLink/>). Nucleotide sequences of primers, conditions used for PCR, and the putative intron amplified are provided with each GenBank dbSTS database entry. Amplification products were visualized by agarose gel electrophoresis, purified, and sequenced to confirm amplification of the targeted region. PCR amplification was then performed using genomic DNA from sires of the USDA bovine reference population (Bishop *et al.* 1994) and products were sequenced to identify single nucleotide polymorphisms (SNP) to be used as markers for linkage mapping. SNP marker genotypes from progeny of informative sires were characterized by direct sequencing using the CEQ8000 or an ABI Prism 3700 DNA automated sequencer (Applied Biosystems, Foster City, CA, USA). Linkage analysis was performed using CRI-MAP v. 2.4 (Green *et al.* 1990). For physical mapping of ER $\beta$ , the Roslin-Cambridge 3000-rad bovine/hamster radiation hybrid panel (Invitrogen Corp.) was used, as previously described (Connor *et al.* 2004).

#### ER $\alpha$ and PR mRNA sequencing

Clones containing partial cDNAs for ER $\alpha$  and PR were identified in two bovine cDNA libraries produced in our laboratory. The PR clone was obtained from a mammary gland library (Sonstegard *et al.* 2002) and the ER $\alpha$  clone was isolated from an intestinal library (Baumann *et al.* 2005). Plasmid DNA was purified and sequenced in one direction first using the primer 5'-TGAGCGGATAACA ATTTACACACAG-3' and DTCS Quickstart chemistry (Beckman Coulter). Gene-specific primers were then designed for sequencing of the entire clone inserts using sequence information obtained from previous reactions (primer walking).

#### Statistical analyses

RT-PCR data were analyzed using a one-way ANOVA. Bonferroni's multiple comparison test was used for



**Figure 1** Expression of transcripts for estrogen receptor alpha (A), progesterone receptor (B), estrogen receptor beta (C), and estrogen-related receptor alpha (D) in bovine mammary gland. Calf, 3-month-old calves ( $n=3$ ); PG, primigravid cows 125–220 days of gestation ( $n=9$ ); LNP, lactating non-pregnant cows, 120 days of lactation ( $n=5$ ); LP, lactating pregnant cows, 240 days of lactation ( $n=5$ ); DP, non-lactating pregnant cows, 20 days after cessation of milking and 40 days before expected parturition ( $n=5$ ). Means without a common superscript differ ( $P<0.05$ ). Note different concentrations on the y axes.

post ANOVA comparisons (Prism, version 4; GraphPad Software Inc., San Diego, CA, USA). Pearson correlation coefficient was determined using the mean transcript quantities for each of the five physiological stages (Prism, version 4).

## Results

### Expression of steroid receptor transcripts

The quantity of transcripts for ER $\alpha$ , ER $\beta$ , PR and ERR $\alpha$  during mammary gland development and the lactation cycle are depicted in Fig. 1. For ER $\alpha$  and PR, transcripts were present in greatest abundance during the prepubertal period. Concentrations declined during first pregnancy, with generally greater concentrations found during late lactation and during the non-lactating period between successive pregnancies; we have referred to this period as the period of regenerative involution (Capuco *et al.* 2003). Following first conception, transcripts for ER $\alpha$  were at the greatest concentration in lactating, pregnant animals and transcripts for PR were greatest during regenerative involution. Expression of ERR $\alpha$  mRNA was moderate during

all stages of development, with lowest concentrations occurring in early lactation. In contrast, ER $\beta$  transcripts were present at very low concentration throughout all stages evaluated (note different concentrations on the y axes in Fig. 1). On average, concentrations for ER $\beta$  were 8- to 190-fold lower than those for ER $\alpha$ , PR and ERR $\alpha$ . Patterns of expression appeared similar for ER $\alpha$  and PR, as did those for ER $\beta$  and ERR $\alpha$ . Correlation coefficients were 0.90 for the relationship between transcript levels of ER $\alpha$  and PR ( $P<0.05$ ) and 0.87 for the relationship between ER $\beta$  and ERR $\alpha$  ( $P<0.05$ ).

### Steroid receptor protein expression

Protein expression was evaluated by IHC (Figs 2 and 3). At all stages, ER $\alpha$  and PR were detected in nuclei of mammary epithelial cells. In the mammary parenchyma of the prepubertal heifer, ER and PR were mainly localized to nuclei of an intermediate layer within the epithelium. In the mature cow, expression of ER $\alpha$  protein was quite uniformly expressed among epithelial cells of the lactating mammary gland, but expression was scattered among epithelial cells during pregnancy and during the period of

regenerative involution. Progesterone receptor protein was expressed among scattered epithelial cells during pregnancy, lactation and regenerative involution. Expression of ER $\beta$  protein could not be detected in mammary tissue at any stage of development or function, although it was detectable in cells of the bovine anterior pituitary (Fig. 3).

### Mapping of steroid receptor genes

To determine location in the cattle genome and to support identity of the bovine hormone receptor sequences according to human gene annotation, the ER $\alpha$ , ER $\beta$ , PR and ERR $\alpha$  genes were mapped to bovine chromosomes 9, 10, 15 and 29 respectively on the USDA linkage map (Table 2). The GenBank accession numbers of each gene-associated sequence tagged site used to identify SNP for linkage analysis, as well as radiation hybrid mapping of ER $\beta$ , are provided in Table 2. The ER $\alpha$  gene was positioned near microsatellite marker BMS2295, at approximately 98.6 cM on the USDA linkage map. The ER $\beta$  gene was positioned at approximately 79.0 cM, near marker INRA037 on the linkage map and near marker INRA096 (distance=0.17 cR; LODS=11.31) on the physical map. The PR gene was mapped near marker BMS2533 at approximately 13.9 cM and ERR $\alpha$  was positioned near marker HH22 at approximately 41.6 cM. The nucleotide sequence data reported in this paper were submitted to GenBank and assigned accession numbers BV166870–166875, AY656812–656813.

### Discussion

ER $\alpha$  was previously mapped to bovine chromosome 9 using somatic cell mapping (Womack *et al.* 1991). In the present study, ER $\alpha$  was positioned towards the telomeric end of bovine chromosome 9 at approximately 98.6 cM on the linkage map. This is the first report of the chromosomal locations of ER $\beta$ , ERR $\alpha$  and PR in cattle. For all four receptors, our findings agree with predicted locations based on bovine–human comparative maps (<http://locus.jouy.inra.fr/>).

In the current work, two cDNA library clones containing partial codes for ER $\alpha$  and PR mRNA were sequenced and submitted to GenBank. For ER $\alpha$ , over 3300 bp of the mRNA sequence not previously available for the bovine were obtained and correspond to the 3'UTR of the transcript. Within this 3'UTR, the bovine sequence shares 91% identity with ovine ER $\alpha$  (accession no. AY033393.1). For PR, approximately 1500 bp of the bovine mRNA sequence were determined and they correspond to putative exons 1 through 8 of the PR gene. The bovine PR nucleotide sequence shares 97% identity with ovine and 92% identity with canine and human mRNA sequences. It provides over 300 bp of PR mRNA sequence not previously available for cattle.

Two major forms of ER exist, the ER $\alpha$  and ER $\beta$  isoforms. We have demonstrated that the predominant isoform in mammary tissue of dairy cows is ER $\alpha$ . The ER $\alpha$  transcript copy number was high throughout the mammary gland life cycle, with decreased expression during gestation, presumably due to high levels of endogenous estrogens. Additionally, the ER $\alpha$  protein was detectable by IHC during all stages examined. In contrast, transcripts for ER $\beta$  were present in exceedingly low concentration and the protein was undetectable by IHC throughout all physiological stages. Similarly, Schams *et al.* (2003) showed low expression levels of ER $\beta$  transcripts and low, but detectable, levels of protein expression by Western blot. These data suggest a relative lack of a role for ER $\beta$  in bovine mammary gland development and lactation.

ER $\alpha$  appears to mediate ductal growth and morphogenesis in mice (Bocchinfuso & Korach 1997) rather than the ER $\beta$  isoform (Couse & Korach 1999). The predominance of ER $\alpha$  prepubertally strongly suggests that the same is true in the bovine mammary gland. The concentration of ER $\alpha$  mRNA was greatest during the prepubertal period of mammary development, when estrogens are present at very low concentrations in the circulation, yet the mammary gland is hormonally responsive. We previously demonstrated that ER $\alpha$  is expressed in a subpopulation of epithelial cells and suggested that the estrogen signal is propagated in a paracrine fashion (Capuco *et al.* 2002a). Paracrine mediation of estrogen-induced mammary ductal growth similarly appears operative in mouse mammary gland. Tissue transplantation studies of epithelium and stroma between ER $\alpha$ -null mice and wild-type mice demonstrated that estrogen responsiveness is mediated by paracrine factors, and that the ER $\alpha$  response differs with developmental stage. In neonatal mice, ER $\alpha$  is expressed solely in the mammary stroma (Shyamala *et al.* 2002) and stromal ER $\alpha$  is necessary and sufficient to induce ductal growth (Cunha *et al.* 1997, Bocchinfuso *et al.* 2000). In adult mice ER $\alpha$  is expressed in a proportion of mammary epithelial and stromal cells (Shyamala *et al.* 2002), and ER $\alpha$  in epithelial cells appears important for tissue response to estrogens (Mueller *et al.* 2002). In both bovine and human mammary gland, paracrine mediation of estrogenic effects seemingly emanate from the limited number of ER $\alpha$ -positive epithelial cells (Capuco *et al.* 2002a, Anderson & Clarke 2004).

During pregnancy, lactation and regenerative involution, expression of ER $\alpha$  appears limited to mammary epithelial cells. The presence of ER $\alpha$  throughout phases of mammary gland development suggests that the potential for responsiveness to estrogenic stimuli exists throughout the life span of the dairy cow.

As with ER $\alpha$ , PR was present in bovine mammary glands during all physiological stages examined. Its expression is limited to mammary epithelial cells and it

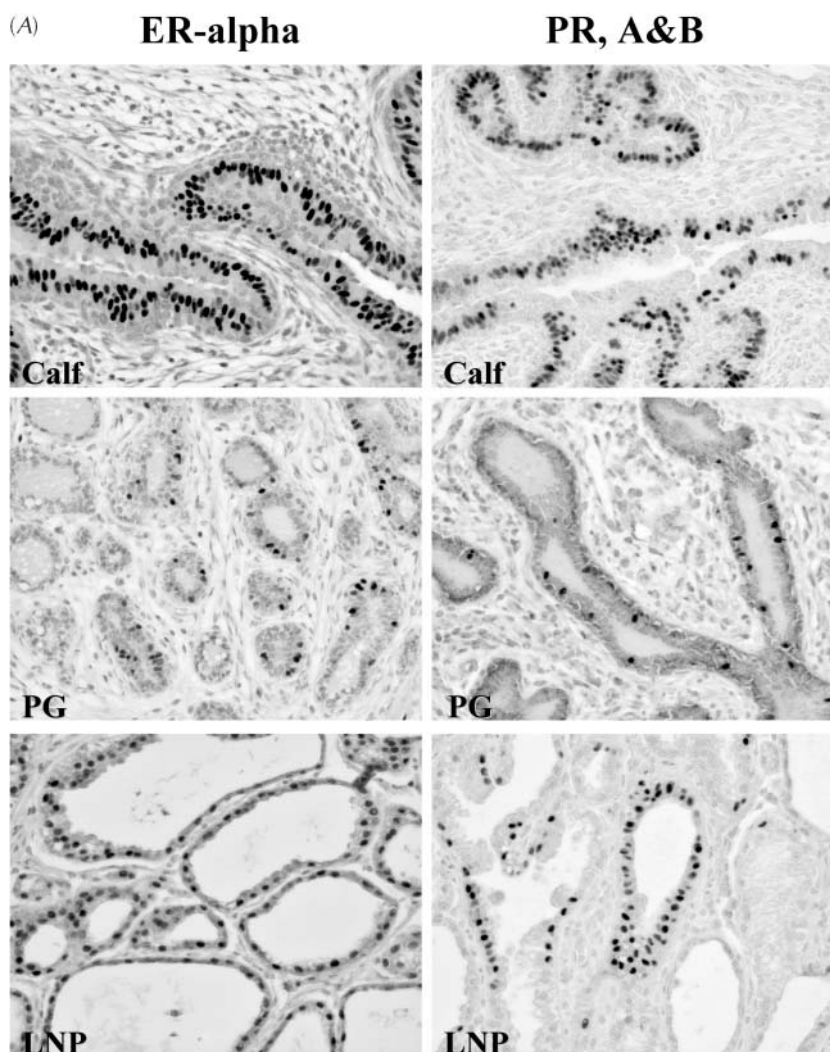


Figure 2 (A).

co-localizes with ER $\alpha$  in cow and human (Press & Greene 1988, Anderson *et al.* 1998, Bartow 1998, Capuco *et al.* 2002a). The primary role of progesterone during mammary development is promotion of lobulo-alveolar development mediated by the PR (Lyons 1958, Nandi 1958, Smith *et al.* 1987, Shyamala 1999). Receptor knockout studies showed that PR in mammary epithelium is essential for ductal branching and lobulo-alveolar development, but not for mammary ductal elongation (Lydon *et al.* 1995). Steroid receptor knockout studies, antiestrogen implantation studies (Silberstein *et al.* 1994) and *in vitro* studies (Haslam & Lively 1985, Woodward *et al.* 1998) suggest that ovarian steroids act directly at the level of the mammary gland. As with estrogen, the effects of progesterone on mammary development appear to involve paracrine mediators (Briskin *et al.* 1998).

Progesterone plays an important role as an inhibitor of the final stage of lactogenesis (Kuhn 1969, Turkington & Hill 1969), yet it does not inhibit milk secretion during an established lactation (Herrenkohl 1974). In mature cows, these effects may be mediated by PR or interactions with other receptors, such as the glucocorticoid receptor (Capuco & Tucker 1980). Assuming that PR serves as mediator, the lack of progesterone inhibition of milk secretion during lactation in rodents may be explained by the absence of the receptor during lactation, whereas its effectiveness as an inhibitor of lactogenesis is consistent with the presence of PR during pregnancy (Haslam & Shyamala 1979). However, we previously demonstrated that PR is expressed in bovine mammary tissue both pre- and post-partum and attributed progesterone's ability to inhibit lactogenesis, but not an established lactation, to the

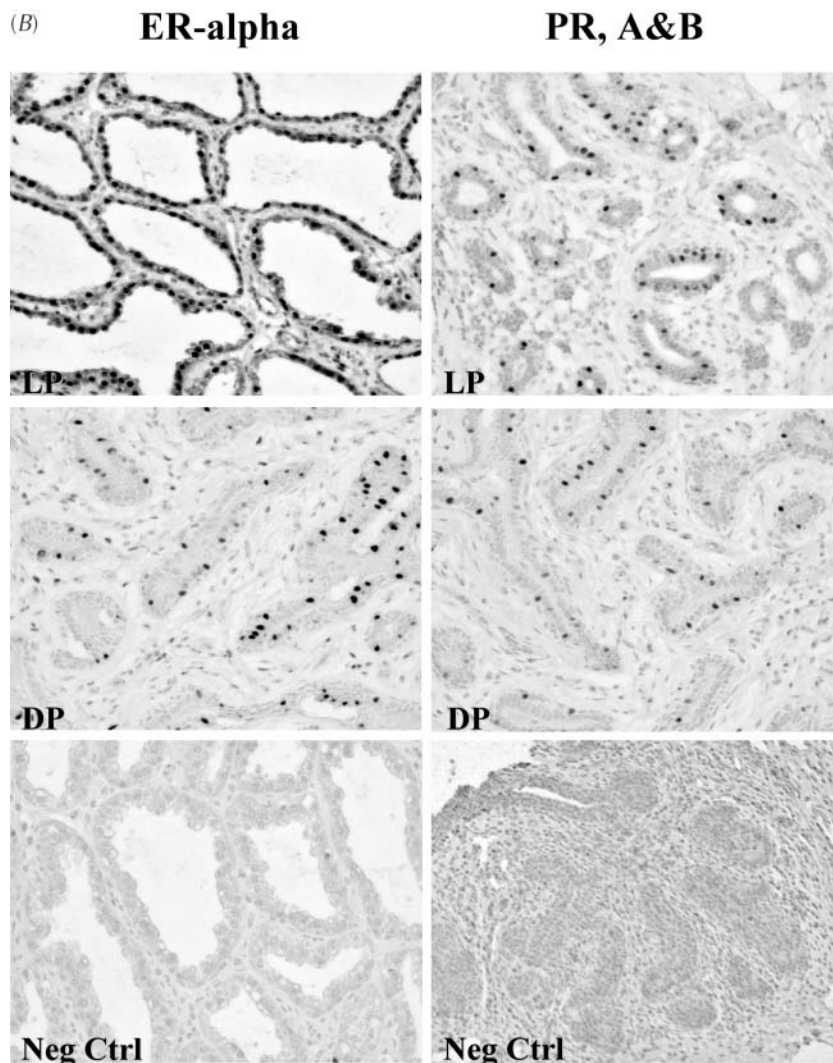


Figure 2 (B).

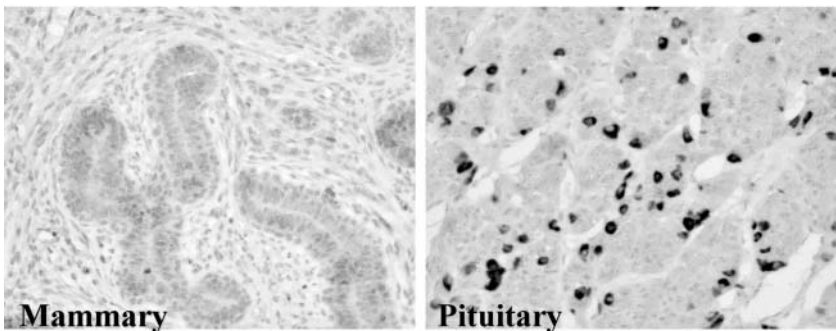
**Figure 2** Immunohistochemical localization of estrogen receptor alpha and progesterone receptor (PR, A&B) in bovine mammary gland. Representative micrographs are shown for (A) mammary glands of 3-month-old calves (calf), primigravid cows 150 days of gestation (PG), and lactating non-pregnant cows, 120 days of lactation (LNP) and (B) lactating pregnant cows, 240 days of lactation (LP) and non-lactating pregnant cows, 20 days after cessation of milking and 40 days before expected parturition (DP; dry pregnant). Negative control slides were incubated with non-immune serum or affinity purified immunoglobulins and showed no staining. Representative negative controls (Neg Ctrl) are shown.

observed decline in PR quantity and a shift in expression of PR isoforms (Capuco *et al.* 1982). Expression of PR in lactating tissues has been confirmed, and PR isoforms have been shown to be differentially expressed in mammary tissue of pregnant versus lactating cows (Schams *et al.* 2003). It is clear that expression of PR is not precluded by the lactational state. Even in mice, PR was expressed in lactating tissue when animals were concurrently pregnant (Capuco *et al.* 2002b). The present study suggests that

expression of PR mRNA and immunoreactive protein during lactation is enhanced by concomitant pregnancy ( $P < 0.1$ ).

We speculate that the greater expression of PR during lactation in cows compared with rodents may be due to differences in ER $\beta$  expression between these species. Expression of PR is induced by activated ER $\alpha$  (Shyamala & Ferenczy 1982, Shyamala *et al.* 1990). However, in rodents ER $\alpha$  does not induce PR during lactation

ER-beta



**Figure 3** Immunohistochemical localization of estrogen receptor beta in mammary gland and anterior pituitary. The mammary gland was devoid of staining for estrogen receptor beta, whereas the pituitary demonstrated positive staining. Tissue sections from the mammary gland of a prepubertal (3-month-old) heifer and from the anterior pituitary of a multiparous nonlactating, nonpregnant cow are depicted.

(Shyamala & Ferenczy 1982), and recent data suggest that this is due to inhibition by ERβ (Saji *et al.* 2001). An ERβ variant with a 54-bp insert between exons 5 and 6 has been reported for rat and referred to as ERβ2 or ERβins (Petersen *et al.* 1998, Saji *et al.* 2001). The ERβins co-localizes with ERα and is in greater abundance than ERα during lactation (Saji *et al.* 2001). The increased ratio of ERβins to ERα during lactation has been hypothesized to permit ERβins to act as a dominant repressor of ERα and to repress expression of PR during lactation (Saji *et al.* 2001). Primers used in the present study amplified a region in the 3' end of exon 8 and would not have distinguished between these variants. Limited expression of ERβ in the bovine mammary gland may preclude repression of ERα activity by ERβ or its variants, resulting in PR expression in the lactating bovine mammary gland.

Based upon sequence homology to ERs, ERRα was the first orphan nuclear receptor to be identified and cloned (Giguere *et al.* 1988). Subsequently, related members (ERRβ and ERRγ) were cloned and studied to a limited extent (Giguere 2002). The ERRs do not bind natural estrogens and activate transcription constitutively (Giguere 2002). ERRα is regulated by estrogens and can regulate estrogen responsive genes (Shigeta *et al.* 1997a, Bai & Giguere 2003), acting as an activator or inhibitor of gene expression depending upon numerous interactions (Vanacker *et al.* 1999, Giguere 2002, Kraus *et al.* 2002, Tremblay & Giguere 2002). Although ERβ was consistently expressed at very low levels in bovine mammary gland, it is noteworthy that the expression of ERRα and ERβ was correlated.

The effects of ERRα on the mammary gland may include roles in cell differentiation and disease resistance.

**Table 2** Summary of linkage mapping of bovine steroid receptors estrogen receptor alpha (ERα), estrogen receptor beta (ERβ), progesterone receptor (PR), and estrogen-related receptor alpha (ERRα)

	Accession no.	SNP*	Chromosome	Nearest marker	2-pt LOD	Recombination fraction
Locus						
ERα	BV166870	A/G@169	9	BMS2295	4.27	0.05
ERβ	BV166871	C/T@112	10	INRA037	9.03	0.00
	BV166872					
PR	BV166874	G/T@33	15	BMS2533	20.70	0.11
		A/G@89				
		G/T@94				
		A/G@113				
		AT indel @121				
		A/T@154				
ERRα	BV166875					
	BV166873	C/T@129	29	HH22	3.61	0.12

\*Type and nucleotides position of SNP in the dbSTS reference sequence used for linkage mapping.

In addition to development within the mammary gland, there are changes in the number and differentiation status of the epithelial cells with each lactation cycle. *ERRα* is necessary for mitochondrial biogenesis (Vega *et al.* 2000, Puigserver & Spiegelman 2003, Mootha *et al.* 2004), appears to play an important role in energy metabolism (Sladek *et al.* 1997, Vega & Kelly 1997, Luo *et al.* 2003, Schreiber *et al.* 2003), and can impact thyroid hormone regulation of energy metabolism (Giguere 2002). A potential role for *ERRα* in facilitating the rapid mammary mitochondrial biogenesis, cell differentiation and energy partitioning among body tissues remains to be evaluated. It has been established that the gene for lactoferrin, a protein secreted into milk as well as secretions of the nonlactating gland, contains ER response elements and is, at least partially, regulated by *ERRα* (Liu *et al.* 1993, Yang *et al.* 1996, Zhang & Teng 2000, 2001). Lactoferrin exhibits iron-binding capacity and bactericidal activity and aids in maintaining mammary gland health through its nonspecific immune functions.

The relationship between these nuclear receptors and dairy production traits warrants further attention. A quantitative trait locus (QTL) for milk somatic cell score (neutrophil concentration of milk, serves as an index of mastitis) was recently identified on bovine chromosome 29 at approximately 50 cM (Ashwell *et al.* 2004). Thus, *ERRα* is a positional candidate gene influencing somatic cell score based on its role in the regulation of lactoferrin. Despite lack of evidence for a role of *ERβ* in bovine mammary gland development and lactation, *ERβ* may have a significant impact on dairy production through effects on ovarian function. A putative QTL for ovulation rate has been identified on bovine chromosome 10 with a peak at approximately 75 cM (Arias & Kirkpatrick 2004), suggesting *ERβ* as a positional candidate gene contributing to this trait. *ERβ* knockout mice exhibit decreased ovulation rate and reduced fertility (Krege *et al.* 1998), further implicating *ERβ* as a candidate gene controlling fertility of dairy cattle. The relationships between *ERβ* and ovulation, *ERRα* and mastitis, and *ERRα* and mammary gland biology in dairy cattle are worthy of further investigation.

In conclusion, our results demonstrate the presence of *ERα*, *PR* and *ERRα* during all physiological stages of the mammary gland and suggest a functional role for these nuclear receptors throughout the life cycle of the dairy cow. Very low concentrations of *ERβ* suggest a relative lack of a role for this transcription factor in bovine mammary gland development and lactation. Additional research pertaining to the spatial, temporal and functional attributes of *ERRα* expression in the bovine mammary gland is warranted.

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